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— SECTION C —

BOTANICAL SCIENCES

Contents

Page

The Amino Acid Requirements for Pyocyanin Production—
Margaret O. Burton, Blythe A. Eagles, and Jack J. R. Campbell - 121

Studies with *Bacillus polymyxa*. V. Potassium as a Factor in the Production of 2,3-Butanediol from Starch—
H. Katznelson - - - - - - - - - - 129

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THE AMINO ACID REQUIREMENTS FOR PYOCYANIN PRODUCTION[1]

BY MARGARET O. BURTON,[2] BLYTHE A. EAGLES,[3] AND JACK J. R. CAMPBELL[4]

Abstract

A medium consisting of acid-hydrolysed casein, glycerol, and a salt mixture has been shown to yield pyocyanin by *Pseudomonas aeruginosa* equal in amount to that obtained from glycerol peptone agar. The monoaminomonocarboxylic acids fraction obtained from casamino acids (Bacto) has been shown to be the source of nitrogen essential to pigment formation. Glycine, *dl*-alanine, *dl*-valine, or *l*-tyrosine have been shown to produce pyocyanin when employed as sole sources of nitrogen. The addition of *l*-leucine to media containing glycine or *dl*-alanine markedly increased pyocyanin formation. The addition of *dl*-phenylalanine to a synthetic medium inhibited pigment production. A synthetic medium consisting of *dl*-alanine or glycine at 0.4% concentration combined with 0.8% *l*-leucine, 1.0% glycerol, and a salt mixture has been shown to be the most suitable medium for pyocyanin production by five representative strains of *Pseudomonas aeruginosa*.

Various synthetic and semisynthetic media have been used in studies of pyocyanin production by *Pseudomonas aeruginosa*. Gessard (2) showed that glycerol peptone agar provided an admirable medium for pyocyanin formation. Jordan (3) reported that the ammonium salts of succinic, lactic, acetic, or citric acids could serve as adequate carbon and nitrogen sources for pyocyanin production. Lourié (4) found that acetate, although suitable for the production of the fluorescent pigment would not support pyocyanin development. Robinson (6) stated that the yield of pyocyanin by *P. aeruginosa* from various synthetic media had never been found to equal that from Bacto peptone. Seleen and Stark (7), in studies on the nutritive requirements of *P. aeruginosa*, were unable to devise a medium more suitable for pyocyanin production than the glycerol peptone agar of Gessard.

The object of the present investigation was to ascertain whether amino acids could replace peptone for the production of pyocyanin and to determine which are essential to pigment formation.

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Contribution from the Department of Dairying, The University of British Columbia, Vancouver, B.C. This study was carried out under a grant from the National Research Council of Canada, laboratory facilities being found by The University of British Columbia.

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Methods

The organism employed in this work was *P. aeruginosa* A.T.C. 9027.

A preliminary study of various media showed that formation of pyocyanin equal to that produced on glycerol peptone agar could be obtained when the following medium was employed:

Casamino acids, 1.0% (Bacto acid-hydrolysed casein)
Glycerol, 1.0%
Salt Solutions *A* and *B*,* 0.2 ml. of each per 100 ml. medium
pH, 7.2

Agar was found not to be essential for maximum pyocyanin formation if the medium was aerated during growth in tubes or if a thin layer of the medium in flasks was employed (10 ml. medium in 125 ml. Erlenmeyer flasks). The latter procedure gave more reproducible results. All cultures were incubated at 30° C. for four days.

For the determination of the amino acids essential to pigment formation, the general technique of Mueller (5) was employed. The casamino acids were separated by the Dakin method (1) into three fractions:

I. Monoaminomonocarboxylic acids
II. Proline
III. Diamino- and dicarboxylic acids

These fractions were employed as the source of nitrogen replacing the casamino acids used in the medium described above.

The extent of pyocyanin production was measured by means of the Fischer Electrophotometer. Ten millilitres of culture were shaken with four successive 2 ml. portions of chloroform. The red acid pyocyanin was separated from chloroform by the addition of 5.0 ml. of 0.1 *N* hydrochloric acid. Five millilitres of 0.1 *N* sodium hydroxide was then added and the resulting blue pyocyanin solution was diluted with an equal volume of distilled water. The density of pigment was measured electrophotometrically. The effect of dilution of pigment on photoelectric readings was found to be a linear relationship.

Experimental

Fractions I, II, and III, recombined and added to the basal medium of glycerol and salts in the following amounts: Fraction I, 0.5 gm., Fraction II, 0.18 gm., and Fraction III, 0.63 gm. per 100 ml., resulted in pyocyanin formation equal to that obtained when the unfractionated casamino acids were employed as the source of nitrogen.

In order to determine which of the fractions were essential to pyocyanin production, media whose nitrogen sources consisted entirely of individual

* *Salt Solution A: dipotassium hydrogen phosphate, 0.05 gm. per ml.*
potassium dihydrogen phosphate, 0.05 gm. per ml.
Salt Solution B: magnesium sulphate septahydrate, 0.05 gm. per ml.
potassium chloride, 0.02 gm. per ml.
ferrous sulphate septahydrate, 0.0002 gm. per ml.

fractions, or of various combinations of the respective fractions at different percentage concentrations, were prepared. Glycerol and Salt Solutions *A* and *B* at the percentages employed in the preliminary study were used throughout. Table I lists the media used and the amount of pyocyanin produced from each medium.

TABLE I

THE EFFECT OF FRACTIONATED CASAMINO ACIDS ON PYOCYANIN FORMATION

Medium*	Fractions of casamino acids	Pyocyanin**
1*a*	I (0.5%)	29.3
2*a*	II (0.18%)	0
3*a*	III (0.63%)	0
4*a*	I (0.5%) + II (0.18%)	31.5
5*a*	I (0.5%) + III (0.63%)	68.7
6*a*	II (0.18%) + III (0.63%)	0
7*a*	I (0.5%) + II (0.18%) + III (0.63%)	67.2
1*b*	I (1.0%)	75.5
2*b*	II (1.0%)	0
3*b*	III (1.0%)	0
4*b*	I (0.74%) + II (0.26%)	70.5
5*b*	I (0.44%) + III (0.56%)	51.7
6*b*	II (0.22%) + III (0.78%)	0
Casamino acids (1.0%)		61.0

* *Basal medium: glycerol, 1.0%; Salt Solutions A and B, 0.2 ml. of each per 100 ml. medium.*

** *Pyocyanin expressed as a reading on logarithmic scale of Fischer Electrophotometer.*

It is seen from Table I that when Fraction I is omitted from a medium no pyocyanin formation occurs. When this fraction is present to the extent of 1.0%, even though it is the sole source of nitrogen, pyocyanin production equal to that obtained when unfractionated casamino acids are employed at 1.0% concentration is obtained. At lower concentrations (0.5%) Fraction I employed as the sole source of nitrogen resulted in pyocyanin formation but in smaller amounts. The addition of Fraction II or III to the lower concentrations of Fraction I resulted in formation of increased amounts of pyocyanin. Fractions II and III stimulate the production of pyocyanin in the presence of the lower concentration of Fraction I by providing additional carbon and nitrogen necessary for growth. Thus there is a greater yield of pyocyanin obtained in Medium 4*a* than in Medium 1*a* and a still greater increase in Media 5*a* and 7*a*. Although in these media the percentage of Fraction I remains constant, there is an increase in the carbon and nitrogen contents parallel with the increase in pyocyanin formed. Medium 1*b* contains the amino acids essential to maximum pigment formation in addition to sufficient carbon and nitrogen for the growth requirements of the organism.

A medium made up of the nine monoaminomonocarboxylic acids present in Fraction I and combined in the proportions in which they are present in

casein to total 1.0% was next prepared. The composition of this medium, designated as Medium *A*, is given below:

Medium *A**

Amino acids in Fraction I	Percentage in casein	Equivalent proportion to total 1.0% (1 gm./100 ml.)
Glycine	0.45	0.011
dl-Alanine	1.85	0.046
dl-Valine	6.2	0.155
dl-Isoleucine	6.4	0.16
l-Leucine	9.9	0.248
dl-Phenylalanine	5.2	0.13
l-Tyrosine	5.7	0.14
dl-Serine	0.5	0.016
dl-Threonine	4.2	0.105
	40.5%	1.01 gm.

** Basal medium: glycerol, 1.0%; Salt Solutions A and B, 0.2 ml. of each per 100 ml. medium.*

Nine different media, identical with Medium *A* except for the omission of one of the respective amino acids constituting Fraction I, were also prepared. A medium consisting of Fraction I at 1.0% concentration served as a control. The amounts of pyocyanin produced from these media are presented in Table II.

TABLE II

THE SUBSTITUTION OF FRACTION I BY THE MONOAMINO-MONOCARBOXYLIC ACIDS OF CASEIN

Medium	Constituents of medium	Pyocyanin
A	Nine amino acids of Fraction I	57.0
1	Medium *A* minus glycine	38.0
2	" " " alanine	46.7
3	" " " valine	34.7
4	" " " isoleucine	34.5
5	" " " leucine	34.0
6	" " " phenylalanine	67.0
7	" " " tyrosine	54.0
8	" " " serine	51.0
9	" " " threonine	34.0
Control	Fraction I (1.0%)	77.0

The results show that a synthetic mixture of the nine amino acids as a source of nitrogen was satisfactory for pyocyanin production and that more than one amino acid is concerned with pigment formation. The results obtained when phenylalanine was omitted from the medium suggest that the presence of this amino acid may exert an inhibitory effect on pyocyanin production.

In order to study the effect of individual amino acids on the microbial synthesis of pyocyanin, nine media were prepared, employing each of the respective amino acids of Fraction I as the sole source of nitrogen. Except in the case of tyrosine, which was used at 0.6% concentration, the amino acids were made up at 1.0%. The results of this experiment are recorded in Table III.

TABLE III

PYOCYANIN PRODUCTION FROM INDIVIDUAL AMINO ACIDS OF FRACTION I

Medium	Pyocyanin
1. Glycine, 1.0%	25.5
2. *dl*-Alanine, 1.0%	38.0
3. *dl*-Valine, 1.0%	29.0
4. *l*-Leucine, 1.0%	0
5. *dl*-Isoleucine, 1.0%	0
6. *dl*-Threonine, 1.0%	0
7. *dl*-Phenylalanine, 1.0%	0
8. *dl*-Serine, 1.0%	0
9. *l*-Tyrosine, 0.6%	18.5

While growth of *P. aeruginosa* was obtained in all media employed, pyocyanin production occurred only in the presence of glycine, alanine, valine, or tyrosine as the source of nitrogen. Alanine gave the highest yield of pyocyanin which, however, did not equal the amount obtained when either casamino acids or combinations of the amino acids were employed. Even when alanine was employed up to a concentration of 2.0% no greater yield of pyocyanin was obtained.

With the object of determining the amino acids essential to maximum production of pyocyanin, media whose nitrogen sources consisted of various combinations of the pyocyanin-producing and non-pigment-forming amino acids of Fraction I were prepared. The media used in this experiment and the results obtained are given in Table IV.

Whereas no pigment is formed when leucine is employed as the sole source of nitrogen, the inclusion of this amino acid in a medium containing glycine, alanine, and valine results in a marked increase in pigment production. Leucine is, however, without influence on the pigment-forming ability of tyrosine. The addition of phenylalanine to a mixture of the pigment-forming amino acids results in the complete suppression of pyocyanin, confirming the observation recorded in Table II.

As the results obtained from these experiments showed clearly that the concentrations of the amino acids used in media were of marked significance with respect to the production of pyocyanin, media made up of various concentrations of alanine and leucine were prepared. Fraction I at 1.0% concentration again served as the control medium. The results are given in Table V.

TABLE IV

THE EFFECT OF VARIOUS COMBINATIONS OF MONOAMINOMONOCARBOXYLIC ACIDS ON PYOCYANIN PRODUCTION

	Glycine, %	*dl*-Alanine, %	*dl*-Valine, %	*l*-Tyrosine, %	*l*-Leucine, %	*dl*-Isoleucine, %	*dl*-Threonine, %	*dl*-Phenylalanine, %	*dl*-Serine, %	Pyocyanin
1	0.2	0.2	0.2	—	—	—	—	—	—	21.0
2	0.4	0.4	0.4	—	—	—	—	—	—	40.0
3	0.2	0.2	0.2	—	0.6	—	—	—	—	87.5
4	0.2	0.2	0.2	—	—	0.6	—	—	—	33.5
5	0.2	0.2	0.2	—	—	—	0.6	—	—	53.0
6	0.2	0.2	0.2	—	—	—	—	0.6	—	0
7	0.2	0.2	0.2	—	—	—	—	—	0.6	24.0
8	—	—	—	0.6	—	—	—	—	—	12.0
9	—	—	—	0.6	0.2	0.2	0.2	—	—	10.5
10	—	—	—	0.6	—	—	—	0.2	0.2	0

TABLE V

THE EFFECT OF VARIOUS COMBINATIONS OF ALANINE AND LEUCINE ON PYOCYANIN PRODUCTION

Medium	Alanine, %	Leucine, %	Pyocyanin
1	1.0	—	41.0
2	—	1.0	0
3	1.0	0.2	66.0
4	0.8	0.4	76.0
5	0.6	0.6	68.0
6	0.4	0.8	93.0
7	0.2	1.0	78.0
Control	Fraction I at 1.0% concentration		75.5

TABLE VI

THE STIMULATORY EFFECT OF *l*-LEUCINE ON PYOCYANIN PRODUCTION FROM ALANINE, GLYCINE, AND VALINE

Medium	Constituents of medium	Pyocyanin
1	Alanine, 0.4%	35.5
2	Alanine, 1.2%	43.0
3	Alanine, 0.4% + *l*-leucine, 0.8%	96.0
4	Glycine, 0.4%	18.0
5	Glycine, 1.2%	8.5
6	Glycine, 0.4% + *l*-leucine, 0.8%	95.0
7	Valine, 0.4%	11.5
8	Valine, 1.2%	27.5
9	Valine, 0.4% + *l*-leucine, 0.8%	33.0

The maximum yield of pyocyanin was obtained when 0.4% alanine and 0.8% leucine were incorporated in the medium, surpassing pyocyanin production in the control, Fraction I.

A comparative study of the effect of glycine, alanine, and valine, employed as sole sources of nitrogen at two different concentrations and combined with leucine, on pyocyanin formation is presented in Table VI.

The stimulating effect of *l*-leucine on pyocyanin production is shown to be most marked in the case of glycine and alanine and, as a result of these studies, synthetic media for maximum production of pyocyanin by *P. aeruginosa* A.T.C. 9027 have been developed. Either of the media detailed below yielded pyocyanin in quantities greater than those obtained on any other medium.

1. Glycine, 0.4%
   l-Leucine, 0.8%
   Glycerol, 1.0%
   Salt Solutions *A* and *B*, 0.2 ml. of each per 100 ml. medium

2. *dl*-Alanine, 0.4%
   l-Leucine, 0.8%
   Glycerol, 1.0%
   Salt Solutions *A* and *B*, 0.2 ml. of each per 100 ml. medium

In order to ascertain the general suitability of a synthetic medium for pyocyanin production by *Pseudomonas aeruginosa* it was considered essential to study other representative strains of the species. In Table VII pyocyanin production by five strains on seven different media are compared.

TABLE VII

COMPARISON OF PYOCYANIN PRODUCTION BY DIFFERENT STRAINS OF *P. aeruginosa*

Medium	Constituents of medium	*P. aeruginosa* A.T.C. 9027	*P. aeruginosa* 8689	*P. pyocyaneus* V21	*P. aeruginosa* R.M.	*P. aeruginosa* Pa.*
1	Fractions II and III	0	0	0	0	0
2	Glycine, 1.0%	17.0	12.0	21.5	28.0	16.5
3	*dl*-Alanine, 1.0%	38.0	26.0	30.0	23.0	18.5
4	*dl*-Valine, 1.0%	29.0	25.0	18.5	20.5	9.5
5	*l*-Tyrosine, 0.6%	9.5	10.5	7.5	10.0	8.0
6	*dl*-Alanine, 0.4% *l*-leucine, 0.8%	97.0	50.0	38.5	50.0	18.0
7	Casamino acids, 1.0%	67.0	31.0	25.5	51.0	20.0

* *We are indebted to Dr. M. Scherago, Department of Bacteriology, University of Kentucky, for culture of P. aeruginosa Pa.*

The pyocyanin forming ability of the respective strains of *P. aeruginosa* is shown to vary markedly. The synthetic medium in which alanine and leucine are employed as the source of nitrogen is, however, shown to be equal or superior to casamino acids for pigment formation by all strains studied.

References

1. DAKIN, H. D. Biochem. J. 12 : 290-317. 1918.
2. GESSARD, C. Ann. inst. Pasteur, 4 : 88-102. 1890.
3. JORDAN, E. O. J. Exptl. Med. 4 : 627-647. 1899.
4. LOURIÉ, H. Thesis. The University of British Columbia. 1945.
5. MUELLER, H. J. J. Bact. 29 : 515-530. 1935.
6. ROBINSON, G. L. Brit. J. Exptl. Path. 13 : 310-317. 1932.
7. SELEEN, W. A. and STARK, C. N. J. Bact. 46 : 491-500. 1943.

STUDIES WITH *BACILLUS POLYMYXA*

V. POTASSIUM AS A FACTOR IN THE PRODUCTION OF 2,3-BUTANEDIOL FROM STARCH[1]

By H. Katznelson[2]

Abstract

Potassium either as potassium chloride or dipotassium hydrogen phosphate was found to stimulate production of 2,3-butanediol from starch by *Bacillus polymyxa* and to increase the diol : ethanol ratio. In a casein hydrolysate medium, potassium alone produced this effect; however, in a synthetic (amino acid) medium, phosphorus was found to cause a slight increase in yield of diol especially in the presence of potassium.

Potassium, phosphorus, and magnesium were shown to be required for growth of *B. polymyxa* in a synthetic medium containing glucose, amino acids, and biotin.

By means of 'resting cells' of *B. polymyxa*, acting on glucose, it was demonstrated that potassium specifically stimulated the diol-synthesizing mechanism and that sodium could replace this element partially.

Introduction

In common with other living forms bacteria need potassium for growth although the amounts required vary with different species and strains as is indicated by analysis of their ash (14). The role of this element in metabolism has been studied by many investigators. Miller (9), in summarizing the function of potassium in plants, states that it plays a part in the formation of carbohydrates and in the synthesis of protein, that it acts as a catalyst, and that it appears to accelerate enzymatic activity. Further evidence of its role in carbohydrate and nitrogen metabolism in plants is presented by Nightingale (10) and others (4, 15, 16, 17). The influence of potassium on the activity of respiratory enzyme systems has also received considerable attention (2, 8, 11). For example Lasnitzki (8) states that "potassium is able to activate the enzymatic breakdown of glucose with phosphorylation of glucose possibly the initial reaction". This is supported by Boyer *et al.* (2) who demonstrated that potassium acts in the phosphorylation of the adenylic system by accelerating the transfer of phosphate from 3-phosphoglycerate to creatine. Davies (3) noted that potassium is an essential factor in the fermentation of maize by *Clostridium acetobutylicum*, probably being required for growth. In this paper is reported the influence of potassium on the production of 2,3-butanediol from starch.

Experimental Methods

The procedure employed and materials used are identical with those reported in the preceding paper of this series (7). Inoculum was prepared by centri-

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Contribution No. 236 (Journal Series) from the Division of Bacteriology and Dairy Research, Science Service, Department of Agriculture, Ottawa.

[2] *Associate Bacteriologist.*

fuging a 24-hr. fluid culture, but the cells were washed several times in 0.9% saline prior to final suspension in distilled water in order to reduce carry-over of the elements under investigation to a minimum. Baker's C.P. reagents were used throughout.

Experimental Results*

Starch Fermentation

In Table I are presented results of omitting the salts included in Solutions *A* (dipotassium hydrogen phosphate and potassium dihydrogen phosphate) and *B* (magnesium sulphate, ferrous sulphate, manganese sulphate, and sodium chloride) of Snell and Strong (12) from media containing casein hydrolysate or amino acids. It is clear that the response to inorganic salts is considerably

TABLE I

INFLUENCE OF INORGANIC SALTS ON PRODUCTION OF 2,3-BUTANEDIOL FROM STARCH BY *Bacillus polymyxa*

Strain	Casein hydrolysate medium		Amino acid medium	
	Plus salts	Minus salts	Plus salts	Minus salts
	Diol, %		Diol, %	
39	0.75	0.69	1.10	0.57
47	1.28	0.64	1.38	0.49
82	0.75	0.52	0.98	0.37
252	0.59	0.41	0.74	0.32

greater in the synthetic medium (amino acid) than in the casein hydrolysate medium, which undoubtedly contains various inorganic elements. Strain 47 is most sensitive to salt deficiency even in the casein hydrolysate medium. However, complete suppression of the fermentation was not achieved even with the synthetic medium by omission of these salts owing to the ash content of the starch itself.**

When the individual salts were omitted separately from a medium containing all (with Strain 47 as the test organism) it was found that dipotassium hydrogen phosphate was the only one required for fermentation. Experiments were then carried out with the four strains in an amino acid medium with and without this salt. Analysis† for both butanediol and ethanol were made at three and five days as indicated in Table II. The results show clearly that dipotassium hydrogen phosphate is required by all four strains especially after three days' incubation but that an appreciable yield of diol and ethanol could be obtained after five days in the absence of this salt; this was due without

* *The data presented are representative of the results obtained in repeated experiments.*

** *According to K. A. Clendenning, Division of Applied Biology, National Research Council, Ottawa, this starch contains 0.26% ash.*

† *No correction was made for the small amount of acetylmethylcarbinol present in each of the two fractions determined.*

TABLE II

EFFECT OF DIPOTASSIUM HYDROGEN PHOSPHATE ON BUTANEDIOL AND ETHYL ALCOHOL PRODUCTION FROM STARCH AFTER THREE AND FIVE DAYS' INCUBATION IN A SYNTHETIC MEDIUM

Strain	Three days						Five days					
	Plus K_2HPO_4			Minus K_2HPO_4			Plus K_2HPO_4			Minus K_2HPO_4		
	Diol, %	Ethanol, %	Diol / Ethanol	Diol, %	Ethanol, %	Diol / Ethanol	Diol, %	Ethanol, %	Diol / Ethanol	Diol, %	Ethanol, %	Diol / Ethanol
39	1.09	0.54	2.03	0.57	0.33	1.72	1.25	0.58	2.17	0.92	0.47	1.97
47	1.31	0.60	2.18	0.49	0.28	1.77	1.38	0.58	2.38	1.01	0.47	2.17
82	1.01	0.55	1.85	0.39	0.28	1.39	—	—	—	—	—	—
252	0.71	0.38	1.86	0.30	0.25	1.19	—	—	—	—	—	—

doubt to the slow utilization of the elements in the ash of the starch. The ratio of butanediol to ethanol also shows a marked increase in response to addition of dipotassium hydrogen phosphate after three days' but levels out after five days' incubation. Maximum yields of diol were obtained with 0.2% dipotassium hydrogen phosphate but as little as 0.005% gave almost as high yields.

Since it was still not evident which of the two elements involved, potassium or phosphorus, was more important, or whether both were required, dipotassium hydrogen phosphate was replaced with potassium chloride and disodium hydrogen phosphate and similar results were obtained, as shown in Table III.

TABLE III

INFLUENCE OF POTASSIUM CHLORIDE AND DISODIUM HYDROGEN PHOSPHATE ON FERMENTATION OF STARCH BY *B. polymyxa* (STRAIN 47)

Salts tested*	Casein hydrolysate medium				Amino acid medium			
	Diol, %	Ethanol, %	Diol + ethanol	Diol / Ethanol	Diol, %	Ethanol, %	Diol + ethanol	Diol / Ethanol
None	0.78	0.36	1.14	2.14	0.48	0.27	0.75	1.79
KCl + Na_2HPO_4	1.27	0.53	1.80	2.40	1.24	0.50	1.74	2.45
Na_2HPO_4	0.82	0.37	1.19	2.19	0.54	0.30	0.84	1.82
KCl	1.36	0.60	1.96	2.26	1.08	0.50	1.58	2.13

* *0.1% each.*

It appears that potassium chloride alone is capable of satisfying the requirements of this organism in casein hydrolysate medium but not entirely in the synthetic medium. The influence of disodium hydrogen phosphate is negligible in the former but is evident to a slight extent in the latter in the presence of potassium chloride.

As pointed out earlier, the starch used in the above experiments apparently contains sufficient inorganic elements to support some activity of the organism at three days and appreciable activity at five. A very pure starch,* containing 0.17% ash was then tested in an amino acid medium containing different concentrations and combinations of potassium chloride and disodium hydrogen phosphate as given in Table IV. The data show that addition of potassium

TABLE IV

FERMENTATION OF HIGHLY PURIFIED STARCH AS AFFECTED BY DIFFERENT AMOUNTS OF POTASSIUM AND PHOSPHORUS

Salts tested	Diol, %	Ethanol, %	Diol + ethanol	$\frac{\text{Diol}}{\text{Ethanol}}$
None	0.16	0.12	0.28	1.29
Complete salt solution (*A* and *B*)	0.96	0.43	1.39	2.22
0.005% KCl	0.68	0.36	1.04	1.88
0.01% "	0.81	0.42	1.23	1.93
0.05% "	0.79	0.44	1.23	1.78
0.1% "	1.01	0.45	1.46	2.40
0.1% " + 0.005% Na_2HPO_4	0.91	0.41	1.32	2.28
0.1% " + 0.1% "	0.87	0.39	1.26	2.24
0.1% " + 0.2% "	1.02	0.41	1.44	2.45
0.2% " + 0.2% "	1.05	0.47	1.51	2.25
0.1% Na_2HPO_4	0.17	0.17	0.34	1.03
0.1% " + 0.005% KCl	0.50	0.26	0.76	1.87
0.1% " + 0.2% "	0.95	0.38	1.33	2.47

chloride alone results in maximum yield of diol under the conditions of the experiment. The fermentation of this pure starch was almost negligible at both three and five days in the absence of the potassium salt. Owing also to its greater purity, this starch was not fermented completely even in the presence of dipotassium hydrogen phosphate; this was accomplished only on the addition of yeast extract.

It cannot be concluded from the above evidence that phosphorus is not required in this fermentation. The organism may obtain enough of this element for its metabolism from the starch but certainly does not obtain sufficient potassium. Earle and Milner (5) found that very pure wheat starch contained 0.13% ash and 0.048% phosphorus, an amount that may satisfy the requirements of the organism.

The influence of potassium on the diol : ethanol ratio is again evident. Added phosphorus exerts no such effect.

Growth Studies

The question of the role of potassium in this fermentation naturally arises. Is it required for normal growth of the organism only or does it also specifically catalyse the synthesis of the diol? The influence of various elements on

* *Kindly supplied by K. A. Clendenning, National Research Council, Ottawa.*

growth of *B. polymyxa* Strain 47 was studied in a synthetic medium containing biotin (0.3μ per litre), glucose 1%, and amino acids. Salt Solutions *A* and *B* were tested first and then their component salts omitted as indicated in Table V. It is obvious that components of both Salt Solutions *A* and *B* are required

TABLE V

GROWTH OF *B. polymyxa* AS AFFECTED BY DIFFERENT INORGANIC ELEMENTS

Salts added to basal medium	Light transmission, %*
1. None	100
2. Salt Solutions *A* and *B*	41
3. 2 minus salts in Solution *A* (K_2HPO_4 + KH_2PO_4)	100
4. 2 minus salts in Solution *B* ($MgSO_4$, $MnSO_4$, $FeSO_4$)	80
5. 2 minus $MgSO_4$	80
6. 2 minus $FeSO_4$	44
7. 2 minus $MnSO_4$	45
8. Na_2HPO_4	75
9. Na_2HPO_4 + Salt Solution *B*	67
10. KCl	98
11. KCl + Salt Solution *B*	100
12. KCl + Na_2HPO_4	81
13. KCl + Na_2HPO_4 + Salt Solution *B*	43
14. KCl + Na_2HPO_4 + $MgSO_4$	44

* *Turbidity readings made with Luxtrol colorimeter after 96 hr. incubation; averages of triplicate determinations.*

for good growth. These components proved to be salts containing potassium, phosphorus, and magnesium; potassium chloride, disodium hydrogen phosphate, and magnesium sulphate completely substituted for Salt Solutions *A* and *B*. Since addition of magnesium was not required in the fermentation of starch, and phosphorus required only to a limited extent, it may be assumed that these elements were present in sufficient amounts in the starch to permit normal development of the organism. Such was not the case with potassium.

Resting Cell Studies

The specific influence of potassium on the synthesis of butanediol was further demonstrated by use of resting cells. These were grown in a medium containing tap water, glucose 1%, peptone 0.5%, and yeast extract 0.05%, dispensed in 100 ml. amounts in Erlenmeyer flasks. After inoculation with two drops of a suspension of cells from a 24 hr. agar slant culture, the flasks were incubated for 20 to 24 hr. at 28° C. The cells from 850 ml. of culture fluid were centrifuged and resuspended twice, brought to a volume of 60 ml. in distilled water, and dispensed in 10 ml. amounts into clean 125 ml. Erlenmeyer flasks containing 1.1 gm. glucose, 0.11 gm. calcium carbonate, and 1 ml. (containing 11 mgm.) of the inorganic salt solutions being tested. Where two salts were used they were combined in the same concentration in 1 ml. solution. The flasks were stoppered and incubated at 28° C., a control flask being kept

in the refrigerator. All flasks were shaken at 15-min. intervals for five hours after which those in the incubator were transferred to the refrigerator over night. Fluctuations in results were very wide unless these conditions were observed. On the following day, the contents of all flasks were transferred to centrifuge tubes, rotated one minute to throw down the calcium carbonate, and then aliquots were removed for determination of 2,3-butanediol and ethanol (7). The diol figures given include acetoin, which was present in small amounts. The ethanol figures were corrected for acetoin that may have distilled over, by a separate determination on the distillate. The averages of seven separate experiments are given in Table VI. Metabolic activity of

TABLE VI

INFLUENCE OF POTASSIUM ON PRODUCTION OF 2,3-BUTANEDIOL AND ETHANOL FROM GLUCOSE BY RESTING CELLS OF *B. polymyxa* (STRAIN 47)

Salts tested	Diol, mM	Ethanol, mM	$\frac{\text{Diol}}{\text{Ethanol}}$	Diol + ethanol, mM
None	7.16	4.43	1.62	11.59
K_2HPO_4	14.56	8.13	1.79	22.69
KCl + Na_2HPO_4	13.55	7.45	1.82	21.0
KCl	13.51	7.65	1.77	21.6
Na_2HPO_4	9.53	8.32	1.14	17.85

the cells alone was quite extensive as yield of total products indicates. Disodium hydrogen phosphate stimulated increased activity and synthesis of both diol and ethanol but resulted in a sharp drop in the diol : ethanol ratio. Maximum activity occurred only where potassium was present alone or together with phosphorus. The effect of disodium hydrogen phosphate may have been due to phosphorus but may also have been due to sodium, substituting partially for potassium. Use of resting cells provided a means of testing this point. The effect of sodium was not studied in the growth and fermentation experiments since sodium hydroxide was used to adjust the reaction of the medium. Accordingly, potassium chloride, sodium chloride, and disodium hydrogen phosphate were tested by the procedure outlined above, the resulting diol values being, in millimoles: 12.24, 7.00, and 7.88 respectively; the untreated control gave a value of 4.70 mM. Diol : ethanol ratios were identical for the sodium chloride and disodium hydrogen phosphate series and highest with potassium chloride. These data are evidence, therefore, of the partial substitution of potassium by sodium and accounts for the effect of disodium hydrogen phosphate. Added phosphorus exerts little influence, apparently being present in adequate amounts for respiration, within the cells.

Similar experiments with starch were unsuccessful owing to the very small amounts of diol produced under the conditions described.

Discussion

According to Adams and Stanier (1) the chief products of fermentation of glucose by *B. polymyxa* are 2,3-butanediol, ethanol, carbon dioxide, and hydrogen; in addition, small amounts of acetoin and acetic acid are produced. They assumed too that the normal dissimilatory mechanisms existing in many carbohydrate fermentations are also operative in this fermentation, that is, the carbohydrate breakdown proceeds to a two-carbon compound; butanediol is formed by condensation and ethanol by reduction of this carbon fraction. The work of Stahly and Werkman (13) supports this view in that they found that acetaldehyde, when added to a vigorous culture of *B. polymyxa* caused definite increases in diol, acetylmethylcarbinol, and ethanol. In addition they demonstrated that calcium acetate increased the yield of diol and acetylmethylcarbinol, postulating that acetic acid also plays a part in the biological synthesis of these compounds. It may be first reduced to acetaldehyde, which may condense with itself or pyruvic acid to acetylmethylcarbinol, which is reduced to diol.

It has been shown that potassium caused an increase in yield of both ethanol and butanediol from starch but that the latter accumulated at a faster rate than the former resulting in a higher diol : ethanol ratio. Addition of disodium hydrogen phosphate to the starch medium stimulated little if any activity. However, phosphorus is necessary for growth; consequently it is assumed that the starch contained sufficient phosphorus to permit normal or almost normal growth and fermentation provided potassium was added. Since magnesium is also required for growth it is assumed again that the starch contained sufficient magnesium to satisfy the requirements of the organism.

Attempts to elucidate the effect of potassium were also made using resting cells with glucose as the substrate. Starch did not lend itself to this work under the conditions described. However, the influence of potassium was found to be the same on glucose as on starch, that is, it specifically stimulated diol formation and thus, an increase in the diol : ethanol ratio. These experiments also showed that sodium can substitute partially for potassium, an effect that has been observed by various investigators (6, 9).

Despite the clear-cut evidence of the specific effect of potassium on diol synthesis, however, nothing is known of its actual function in this reaction. Its role as a catalyst or condensing agent in synthetic chemical processes has been recognized by various workers (9). The observed effect on enzyme function may involve a change in the degree of dispersion of the enzyme, in its combining and absorbing power or its degree of dissociation (9). Since potassium is considered "an essential part of the physico-chemical structure of the cell", moving about with considerable freedom, probably in response to acid–base imbalances, it undoubtedly plays a "modifying role in nearly every vital process" (6).

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References

1. ADAMS, G. A. and STANIER, R. Y. Production and properties of 2,3-butanediol. III. Studies on the biochemistry of carbohydrate fermentation by *Aerobacillus polymyxa*. Can. J. Research, B, 23 : 1-9. 1945.
2. BOYER, P. D., LARDY, H. A., and PHILLIPS, P. H. Further studies on the role of potassium and other ions in the phosphorylation of the adenylic system. J. Biol. Chem. 149 : 529-541. 1943.
3. DAVIES, R. Studies on the acetone–butyl alcohol fermentation. III. Potassium as an essential factor in the fermentation of maize meal by *Cl. acetobutylicum* (BY). Biochem. J. 36 : 596-599. 1942.
4. DAY, D. Starch formation in tobacco plants deficient in potassium. Plant Physiol. 15 : 367-375. 1940.
5. EARLE, F. R. and MILNER, R. T. Improvements in the determination of starch in corn and wheat. Cereal Chem. 21 : 567-575. 1944.
6. FENN, W. O. The role of potassium in physiological processes. Physiol. Rev. 20 : 377-415. 1940.
7. KATZNELSON, H. Studies with *Bacillus polymyxa*. IV. Nitrogen requirements in relation to 2,3-butanediol production from starch. Can. J. Research, C, 24 : 99-108. 1946.
8. LASNITZKI, A. Potassium and carbohydrate metabolism. Nature, 146 : 99-100. 1940.
9. MILLER, E. C. Plant physiology. 2nd ed. McGraw-Hill Book Co., Inc., New York. 1938.
10. NIGHTINGALE, G. T. Physiological–chemical functions of potassium in crop growth. Soil Sci. 55 : 73-78. 1943.
11. QUASTEL, J. H. and WEBLEY, D. M. Vitamin B_1 and bacterial oxidations. II. The effects of magnesium, potassium and hexose diphosphate ions. Biochem. J. 36 : 8–33. 1942.
12. SNELL, E. E. and STRONG, F. M. A microbiological assay for riboflavin. Ind. Eng. Chem., Anal. Ed. 11 : 346-350. 1939.
13. STAHLY, G. L. and WERKMAN, C. H. Origin and relationship of acetylmethylcarbinol and 2,3-butylene glycol in bacterial fermentations. Biochem. J. 36 : 575-581. 1942.
14. STEPHENSON, M. Bacterial metabolism. 2nd ed. Longmans, Green & Company, London. 1939.
15. SUGAWARA, T. The influence of potassium on the respiration, enzyme activity, and ascorbic acid content in potato-tubers. J. Sci. Soil Manure, Japan, 15 (3) : 153-164. 1941. *Abstract in* Biol. Abstracts, 17 : 19174. 1943.
16. TIEDJENS, V. A. and WALL, M. E. The importance of potassium in the growth of vegetable plants. Proc. Am. Soc. Hort. Sci. 36 : 740-743. 1938 (1939).
17. WALL, M. E. The role of potassium in plants. III. Nitrogen and carbohydrate metabolism in potassium-deficient plants supplied with either nitrate or ammonium nitrogen. Soil Sci. 49 : 393-409. 1940.

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